Atkinson, C., Ray, R. M., Li, W., Lin, M-G., Gao, D. L., Shannon, J., Stalsberg, H., Porter, P. L., Frankenfeld, C. L., Wähälä, K., Thomas, D. B., & Lampe, J. W. (2016). Plasma equol concentration is not associated with breast cancer and fibrocystic breast conditions among women in Shanghai, China. *Nutrition Research*, *36*(8), 863-871. https://doi.org/10.1016/j.nutres.2016.03.008

Plasma equol concentration is not associated with breast cancer and fibrocystic breast conditions among women in Shanghai, China

Charlotte Atkinson[a,b], Roberta M. Ray[c], Wenjin Li[c], Ming-Gang Lin[c], Dao Li Gao[d], Jackilen Shannon[e], Helge Stalsberg[f], Peggy L. Porter[c], Cara L. Frankenfeld[g], Kristiina Wähälä[h], David B. Thomas[c], Johanna W. Lampe[c]

[a] Bristol Dental School, University of Bristol, Bristol, UK

[b] NIHR Biomedical Research Unit in Nutrition, Diet, and Lifestyle, University Hospitals Bristol NHS Foundation Trust and University of Bristol, Bristol, UK

[c] Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[d] Zhong Shan Hospital Cancer Center, Shanghai, China

[e] Oregon Health & Sciences University, Portland, OR, USA

[f] University of Tromsø, Tromsø, Norway

[g] George Mason University, Fairfax, USA

[h] University of Helsinki, Helsinki, Finland

CORRESPONDING AUTHOR:

Charlotte Atkinson, PhD

NIHR Biomedical Research Unit in Nutrition, Diet and Lifestyle

Level 3, University Hospitals Bristol Education Centre

Upper Maudlin Street, Bristol BS2 8AE

Phone: 011-44-117-3421766, Fax: 011-44-117-3420239, Email: charlotte.atkinson@bristol.ac.uk

ABBREVIATIONS

BC; breast cancer

BSE; breast self-examination

CI; confidence interval

FBC; fibrocystic breast condition

LC; liquid chromatography

LOQ; limit of quantification

MS; mass spectrometry

NCT; non-cancerous tissue

OR; odds ratio

STIB; Shanghai Textile Industrial Bureau

TR-FIA; time-resolved fluoroimmunoassay

ABSTRACT

Equol (a bacterial metabolite of the soy isoflavone daidzein) is produced by 30-50% of humans and may be associated with health outcomes. We hypothesized that plasma equol would be inversely associated with risks of fibrocystic breast conditions (FBC) and breast cancer (BC). Plasma from women in a breast self-examination trial in Shanghai with BC (n=269) or FBC (n=443), and age-matched controls (n=1027) was analyzed for isoflavones. Equol was grouped into categories (<20, 20-<45, and ≥45 nmol/L) and, among women with daidzein ≥20 nmol/L, the $\log_{10}$ equol:daidzein ratio was grouped into tertiles. Where available, non-cancerous tissue (NCT) adjacent to the carcinomas from women with BC were classified as non-proliferative or proliferative (n=130 and 172, respectively). The lesions from women with FBC were similarly classified (n=99 and 92, respectively). Odds ratios (OR) and 95% confidence intervals (CI) were calculated across equol categories and tertiles of $\log_{10}$ equol:daidzein ratio. Equol categories were not associated with FBC or BC ($p>0.05$). For $\log_{10}$ equol:daidzein, compared to controls there were positive associations in the mid tertile for proliferative FBC (OR 2.06, 95% CI 1.08-3.93), BC with proliferative NCT (OR 2.95, 95% CI 1.37-6.35), and all BC regardless of histology (OR 2.37, 95% CI 1.43-3.95). However, trends in ORs with increasing plasma equol values or equol:daidzein ratios were not observed ($p>0.05$). The results of this study do not provide evidence that equol plays a role in the etiology of these breast conditions. However, further work is needed to confirm or refute this conclusion.

KEYWORDS: breast cancer; daidzein; equol; fibrocystic changes in the breast; isoflavone; nested case-control study; women

1. INTRODUCTION

Soy contains the isoflavones daidzein and genistein [1, 2], and is consumed in high amounts in Asian populations [3-5] and in low amounts by Western populations [6]. Isoflavones are structurally similar to mammalian estrogens [7] and research has focused primarily on their effects on hormone-related conditions, including risk of breast cancer. However, associations between soy or isoflavone consumption and breast cancer risk have been inconsistently observed [8]. Reasons for such differences are unclear but one reason may be due to inter-individual differences in isoflavone metabolism.

Gut microbiota are involved in the metabolism of daidzein to equol [9] and, following soy consumption, approximately 30-50% of individuals produce equol (discussed in [10]). In vitro, equol was shown to have greater biological activity than daidzein, and to have a higher effective free fraction in serum than genistein and 17β-estradiol (discussed in [10]). Thus, it has been suggested that individuals ability to produce equol be considered in studies assessing soy intake and health [11].

Two small studies in Asian and Asian-American populations have shown, albeit non-significantly, lower excretion of equol or a lower proportion of equol-producers than non-producers in breast cancer cases than controls [12, 13], suggesting decreased risk of breast cancer in equol-producers. Similar findings were shown in Western populations [14-16], although one study initially reported an increased risk [17] that attenuated with a larger sample size [18]. Among Chinese immigrant women in the US, mammographic breast density (a marker of risk

for breast cancer) was lower (representing lower risk) in equol-producers than non-producers, and when stratified on equol-producer status, isoflavone intake was inversely associated with breast density among equol-producers but not non-producers [19]. Further, in a cross-sectional study of predominantly White postmenopausal women in the US, there was a suggestion of a favorable interaction between soy intake and equol-producer status on breast density [20].

Proliferative fibrocystic breast conditions (FBC) have been associated with increased risk of breast cancer [21, 22]. We showed previously that plasma genistein and daidzein concentrations were inversely associated with risk of breast cancer and benign FBC among women in Shanghai, China [23]. Associations between equol and these breast conditions remains largely unknown and was the focus of this study. We hypothesized that, in this same population, plasma equol would also be inversely associated with risk of breast cancer and benign FBC. Our specific objectives were to 1) examine associations between equol and risk of breast cancer and benign FBC, and 2) examine these associations stratified by proliferative status of the lesions from women with FBC and of the adjacent non-cancerous tissue of breast cancer cases. Another objective was to confirm whether our previously reported inverse associations between plasma genistein and daidzein concentrations and risk of breast cancer and benign FBC would remain when assessed in a larger sample. We tested these objectives using a case-control study design that was nested within a large trial of breast self-examination [24].

2. METHODS AND MATERIALS

2.1 Study Population

266,064 women (ages 30-64 years) who were current or retired employees of the Shanghai Textile Industrial Bureau (STIB) were enrolled in the breast self-examination (BSE) trial between October 1989 and October 1991 and followed for the development of benign and malignant breast disease through July 31, 2000. Briefly, participants in this study were from two nested case-control studies of benign and malignant breast conditions that were conducted sequentially between September 1995 through August 1997 and between September 1997 and July 2000. The overall recruitment of cases and controls has been described previously [23, 25].

The Institutional Review Board of the Fred Hutchinson Cancer Research Center and the Station for Prevention and Treatment of Cancer in the STIB approved the study, in accordance with the assurances of the Office for Human Research Protection of the US Department of Health and Human Services. Informed consent was obtained prior to interview and blood draw.

2.1.1 Case selection

Case selection has been described in detail elsewhere [23, 25]. New cases of breast cancer (BC) and benign breast disease were identified through review of factory medical clinic records and visits to STIB hospitals. As described previously [25] 622 women with histologically confirmed fibrocystic breast conditions (FBC) and 432 with BC were identified. For breast cancer cases

with adequate non-cancerous tissue (NCT) (at least 5 scanning power fields) from their biopsy, the NCT was classified by one pathologist (ML) according to the scheme developed by Stalsberg [26] as: nonproliferative (mild or no ductal hyperplasia and mild or no sclerosing adenosis), proliferative without atypia (moderate or florid ductal hyperplasia or moderate or predominant sclerosing adenosis and no atypia), or atypia (atypical ductal hyperplasia, atypical lobular hyperplasia or moderate apocrine atypia). The lesions from women with FBC (and no breast cancer) were similarly classified if adequate tissue was available.

As detailed elsewhere [27], in-person interviews were conducted primarily before histologic diagnosis. Data collected during the interviews included demographics, medical history, and known and suspected breast cancer risk factors (see [27] for more information). In our previous study [23], women were excluded from analyses if blood was drawn >30 days prior to diagnosis or >30 days from date of interview. For the present study, the time frame was expanded a priori to include samples taken up to 90 days prior to diagnosis, given that most individuals maintain producer/non-producer phenotypes over time and assignment of phenotypes is unlikely to be influenced by timing of sampling. This resulted in the inclusion of two additional samples (taken at 40 and 47 days prior to diagnosis). Samples drawn within 14 days after diagnosis were included. Interviews were completed for 551 women with FBC (89%), and 443 of these (81%) had plasma that was analyzed for equol (49 samples had been drawn after diagnosis). 302 (68%) of these had histologic classifications as described. Interviews were completed for 378 (88%) women with BC, and equol was measured in plasma from 269 (71%) women (23 samples had been drawn after diagnosis). Of these, 191 (71%) had sufficient NCT for histologic classification.

2.1.2 Control selection

Control selection has been described in detail elsewhere [23, 25]. Controls were selected from unaffected women in the BSE cohort and were frequency-matched to cases on age. Between 1995 and 1997 two controls per benign or malignant case (matched to case on age and menstrual status) were recruited for a concurrent study of cell proliferation and were interviewed in their home or factory (see [23, 25] for more detail). 367 of our controls were recruited in this way. For cases enrolled between 1997 and 2000, controls were frequency matched by 5-year age group and hospital affiliation of their factory in a 1:1 case-control ratio to the largest benign or malignant case group in each age stratum. Interviews were completed in their homes or factories for 704 (82%) of the 862 controls (see [23, 25] for more detail). One control whose calculated daily energy intake was >4000 kcal was excluded. Of the 1070 eligible controls, 1027 had a blood sample drawn at interview for analysis.

2.2 Measurement of plasma isoflavones

Plasma was frozen and stored at -70˚C until assayed for equol using Labmaster time-resolved fluoroimmunoassay (TR-FIA) kits (Turku, Finland). This method was used because it allowed the inclusion of participants with small plasma volumes and provided for improved sensitivity over other methods. Batches had similar distributions of cases and controls. Plasma (200 μl) was incubated overnight at 37^{o}C with 0.2 U/ml β-glucuronidase from E. Coli and 15 U/ml sulfatase (Sigma-Aldrich Co., St. Louis, MO) in 200 μl 0.1 M acetate buffer pH 5. Hydrolyzed samples were extracted twice, each with 1.5 ml ether. Ether fractions were dried under a stream of

nitrogen in a 37°C water bath, and the residue reconstituted in assay buffer. Samples were vortexed, left for approximately 30 minutes, vortexed again and then used in the TR-FIA. Fluorescence was measured on the Wallac Victor 2 model 1420 spectrofluorometer (Turku, Finland). Data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Samples with concentrations greater than the highest standard were assayed using a new plasma aliquot, but the sample was diluted in assay buffer after reconstitution. We used an estimated extraction recovery of 80% as per the package insert, and adjusted concentrations accordingly. The inter-assay CV was 14.0%, and the limit of quantification (LOQ) was 0.66 nmol/L. Concentrations below this were reported as 0.33 nmol/L (i.e., half the LOQ), to allow calculation of ratios.

Samples from most women had been analyzed for daidzein and genistein initially by liquid chromatography-coularray method (LC-coularray; 32% of samples) and then by liquid chromatography-mass spectrometry (LC-MS; 68% of samples); see [28] for further details on these methods. As described previously [28], the analysis method was changed from LC-coularray to LC-MS because increased instrument availability at the time meant that LC-MS could be used, which improved assay efficiency and precision of the measurements. For 217 samples that had not already been analyzed, Labmaster TR-FIA kits (Turku, Finland) were used to measure daidzein and genistein (64 samples for both daidzein and genistein; 49 for genistein only; and 104 for daidzein only) because samples with small volumes could be measured with improved sensitivity. Procedures were as described for equol. Daidzein concentrations <0.5 nmol/L were considered below LOQ and assigned the midpoint of 0.25 nmol/L. Genistein concentrations <1.0 nmol/L were considered below LOQ and assigned the midpoint of 0.5

198 nmol/L. Inter-assay CVs were 9.1% for daidzein and 5.0% for genistein.

199

200 **2.3 Statistical Analyses**

201

202 The women had not received a soy challenge prior to blood sampling so a modified version of
203 the Setchell and Cole method [29] was applied to evaluate equol production in relation to risk of
204 FBC and BC. Setchell and Cole showed that serum equol >20 nmol/L distinguished equol
205 producers from non-producers, and the lowest serum daidzein concentration (following soy
206 exposure) was 16 nmol/L. Thus, we used two approaches to characterize equol exposure. First,
207 we grouped plasma equol into three categories (<20, 20-<45, and ≥45 nmol/L). Second, we
208 restricted analyses to women with plasma daidzein ≥20 nmol/L, calculated the ratio of equol to
209 daidzein (to allow for variation in soy intakes and pharmacokinetics/bioavailability), and $\log_{10}$
210 transformed the result, as per Setchell and Cole [29]. This yielded no clear separation of equol
211 producers from non-producers (data not shown), so we categorized the $\log_{10}$ equol:daidzein ratio
212 into tertiles according to distributions among controls. Because we restricted these analyses to
213 women with plasma daidzein ≥20 nmol/L, additional categories (e.g., quintiles) would result in
214 very small numbers of cases per group. To enable comparisons with previous analyses [23], we
215 categorized daidzein and genistein concentrations into quartiles based on distributions among
216 controls. We used conditional logistic regression models to estimate odds ratios (OR) and 95%
217 confidence intervals (CI), and included strata for blood draw year (1995-1996, 1997, 1998-1999,
218 2000-2001) in all models to account for potential dietary changes prior to and during recruitment.
219 ORs were calculated across categories of equol, $\log_{10}$ equol:daidzein ratio, daidzein, and
220 genistein. ORs for the $\log_{10}$ equol:daidzein ratio in its continuous form were also estimated. We

computed the OR of FBC and BC by comparing each case group to the combined control group (the matching of cases and controls from the first study was not retained in the analysis). We also compared malignant and benign case groups to estimate risk of BC relative to FBC.

For women with histologic data, analyses were conducted according to proliferative status of the NCT. Proliferative conditions with and without atypia were combined because the number of women with atypia was small. Age (5-year categories), plasma genistein, and analysis method for genistein were included in multiple logistic models for equol. Logistic models for $\log_{10}$ equol:daidzein were adjusted for age and plasma daidzein analysis method. We evaluated possible confounding effects of multiple factors, including age at first birth, number of live births, total duration of lactation, years of oral contraceptive use, age at first menstrual period, menopausal status, prior breast lump, times breast self-examination performed per year, body mass index, and education as per our previous analysis [27]. None changed the results appreciably (<10% change in the OR of the primary predictor variable) when added individually, and were not included in final models. Tests for trend were performed by entering categorical variables as continuous variables into regression models. All analyses were based on two-tailed probability using SAS version 9.1 (SAS Institute Inc., Cary, NC).

3. RESULTS

Characteristics of BC cases, women with FBC, and controls were similar to those reported in our previous studies of breast conditions in this population (Table 1) [23, 27, 30, 31].

Geometric mean plasma equol concentration among controls, women with FBC, and BC cases, and also by age group are shown in Table 2. Among controls, equol concentrations ranged from below the LOQ to 395 nmol/L, and 77.5% had concentrations <20 nmol/L (Figure 1). Among BC cases, equol concentrations ranged from below the LOQ to 236 (82.9% had concentrations <20 nmol/L) and among women with FBC equol concentration ranged from below the LOQ to 373 nmol/L (81.7% had concentrations <20 nmol/L).

We observed no associations of benign FBC, BC, or risk of BC vs. FBC in relation to categories (i.e., <20, 20-<45, and ≥45 nmol/L) of plasma equol concentration in all women or when stratified by proliferative status of the FBC or NCT (Table 3). Associations of FBC and BC in relation to tertiles of $\log_{10}$ equol:daidzein ratio in women with plasma daidzein concentrations ≥20 nmol/L are shown in Table 4. Proliferative FBC, BC with proliferative NCT (including atypia), and all BC combined (i.e., all women with and without histological classification) were positively associated with the second tertile of $\log_{10}$ equol:daidzein ratio, but trends across tertiles were not observed. Furthermore, no linear trends were observed when considering the ratio as a continuous variable. Findings did not change substantially when restricting analyses to people whose blood sample was drawn at or before diagnosis, although the OR for proliferative FBC for the second tertile of the $\log_{10}$ equol:daidzein ratio was attenuated (OR 1.80, 95% CI

0.92-3.51). Similarly, findings did not change substantially when restricting analyses to cases and controls with daidzein measured by LC-MS, although the ORs (95% CI) for FBC vs. controls for non-proliferative conditions were 0.38 (0.11-1.32) and 0.20 (0.04-1.01) for the second and third tertiles of the $\log_{10}$ equol:daidzein ratio, respectively (p trend 0.04), and proliferative FBC, BC with proliferative NCT, and BC combined (i.e., all women with and without histological classification) were no longer positively associated with the second tertile of the $\log_{10}$ equol:daidzein ratio. However, these findings were based on small numbers of cases.

For risks of FBC and BC in relation to plasma daidzein and genistein, our findings were similar to those reported previously among the slightly smaller sample [23], although findings for genistein in relation to proliferative FBC were attenuated; briefly, the ORs (95% CI) for the highest quartiles (compared to lowest) of daidzein and genistein, respectively, were 0.17 (0.08-0.38) and 0.30 (0.15-0.60) for non-proliferative FBC and 0.32 (0.15-0.67) and 0.55 (0.29-1.08) for proliferative FBC. The corresponding ORs (95% CI) were 0.26 (0.11-0.62) and 0.43 (0.20-0.96) for BC with concurrent non-proliferative NCT and 0.27 (0.11-0.67) and 0.22 (0.08-0.57) for BC with concurrent proliferative NCT.

4. DISCUSSION

In this population-based case-control study, no trends in risks of either BC or FBC (with or without proliferative changes), were observed with increasing or decreasing levels of either plasma equol concentration or the $\log_{10}$ equol:daidzein ratio. Positive associations were seen for women in the mid tertile of the $\log_{10}$ equol:daidzein ratio for proliferative FBC, BC with proliferative NCT, and total BC. However, the absence of a trend across tertiles and no linear trend when considered in its continuous form suggests that these observations do not represent a biological phenomenon. We reject our hypothesis of an inverse association between plasma equol and risks of FBC and BC among women in Shanghai, China. The inverse association previously shown between plasma daidzein and genistein and risk of these breast conditions [23] remained with the larger sample size, although findings were slightly attenuated.

The effects of equol on human health have been examined previously using blood and urine concentrations of equol or dichotomizing on ability to produce equol [10]. To measure equol, individuals must be exposed to sufficient daidzein prior to sampling. However, as noted by Setchell and Cole [29], there have been inconsistencies across studies in, for example, the amounts of soy/daidzein consumed and cut points for assigning equol-producer status. Although we did not see a clear demarcation between equol producers and non-producers using plasma concentrations, we accounted for this in our analyses by applying some of the criteria specified by Setchell and Cole [29]. Despite this, we did not see any consistent associations between equol production and risks of BC or FBC.

Our findings are in agreement with Virk-Baker et al. who reported no associations between equol-producer status (assessed using a soy challenge) and breast pathology, hyperplasia, or breast cancer among US women who had undergone breast biopsies following an abnormal mammogram [32]. In relation to potential modifying effects of equol production on other breast cancer risk factors, there have been suggestions of greater effects of isoflavone supplementation in equol-producers in relation to estrogen-responsive genes [33], or interactions between equol-producer phenotype and soy intake in relation to mammographic density [19, 20]. However, a soy protein intervention study did not show equol-producer status as an effect modifier regarding mammographic density [34] and there was no effect of equol production on urinary estrogen metabolites in soy supplementation studies [35].

It is possible that the lack of associations in this study may have been due to limited numbers of equol-producers or low circulating concentrations. Around 20% of the women had plasma equol concentrations ≥20 nmol/L which is on the lower end of reported proportions of equol-producers [10]. Furthermore, although equol concentration in our study was higher than or similar to plasma concentrations among men and women in studies of different cancer types in the US or Europe [13, 15, 36, 37], it was lower than concentrations in some studies in Japanese and Korean populations [38-40].

Our study has several strengths. It is a large population-based study in women who typically consume soy foods [28], and most blood samples were drawn before diagnosis or treatment. However, blood was drawn from cases at the time of biopsy and women could have modified their diet prior to the hospital visit. Although this could potentially affect overall circulating

isoflavone concentrations, it is unlikely to affect the capacity of gut microbes to metabolize daidzein to equol. In addition, since most of the cases were asymptomatic and biopsies are considered a minor out-patient procedure, it is unlikely that the women altered their diets as a result of their condition. Also, the women were not instructed to make any changes to their habitual activities or diet in preparation for the hospital visit. Another strength of this study is that the available tissue for histological classification was reviewed by one study pathologist.

One limitation of our study is that we did not administer a soy challenge to classify women according to their equol-producing status. As such, we may have misclassified some individuals due to inadequate or inconsistent soy exposure. Since this misclassification would likely have been the same in cases and controls, this would have the effect of underestimating any true relationship, and could be an explanation for the absence of associations in this study. In a previous study of Chinese men and women consuming their usual diet, the number of equol producers more than doubled when a soy challenge was administered, suggesting that even in populations with high habitual levels of soy consumption the number of equol-producers may be underestimated [41]. Furthermore, in our study, equol was assessed at only one time point. Although equol production has been shown to be relatively stable within individuals over time in some studies [42, 43], others have suggested that around 6 to 20% of individuals vary or crossover equol phenotypes over relatively short periods of time [44, 45]. However, the evidence to date suggests there is more often a producer to non-producer shift than vice versa [42-45]. If that is the case, it may be more likely that non-producers rather than producers were misclassified. Another limitation of this study is that plasma equol concentrations reflect short-term intake and may not reflect exposure at the relevant time for the development of proliferative

mammary epithelial changes, or of cancer initiation or progression. In addition, there may have been insufficient statistical power to evaluate associations, especially for analyses including strata with few cases. Also, different methods of isoflavone analysis were used and daidzein concentrations were slightly lower with LC-MS [28]. However, samples from both cases and controls were measured by LC-MS and it is unlikely that any systematic differences were introduced. Nonetheless, we adjusted for isoflavone analysis method in our statistical model, and when restricting analyses to samples measured by LC-MS, findings did not change substantially. Finally, our study was largely restricted to Han Chinese women residing in one industrial city in China, and the results may not be applicable to women of other races or to women living in different social or physical conditions.

In conclusion, the results of this study do not provide evidence that equol plays a role in the etiology of FBC or breast cancer. However, future studies are needed to more fully explore the potential effects of equol production on risks of these breast conditions.

5. ACKNOWLEDGMENT

We thank Wenwan Wang, Xu Wang Hong, and the medical workers; breast self-examination trial workers and interviewers of the Shanghai Textile Industry Bureau for their efforts in collecting data; Drs. Fan Liang Chen, Guan Lin Zhao, and Lei Da Pan for their support of our studies in Shanghai; Terri Watson for assistance with sample identification; Wendy Thomas, JoAnn Prunty, Heather Skor, and Thomas Kalhorn for their technical assistance with the isoflavone analysis; and Jan Kikuchi, for technical and administrative assistance.

GRANT SUPPORT: This work was supported by National Cancer Institute grants R01 CA75332 and R01 CA120560. The funders did not have a role in either the conduct of the research or the preparation of the article.

CONFLICTS OF INTEREST: None

6. REFERENCES

[1] Reinli K, Block G. Phytoestrogen content of foods--a compendium of literature values. Nutr Cancer. 1996;26:123-48.

[2] Horn-Ross PL, Barnes S, Lee M, Coward L, Mandel JE, Koo J, et al. Assessing phytoestrogen exposure in epidemiologic studies: development of a database (United States). Cancer Causes Control. 2000;11:289-98.

[3] Liu Z, Li W, Sun J, Liu C, Zeng Q, Huang J, et al. Intake of soy foods and soy isoflavones by rural adult women in China. Asia Pac J Clin Nutr. 2004;13:204-9.

[4] Kim J, Kwon C. Estimated dietary isoflavone intake of Korean population based on National Nutrition Survey. Nutr Res. 2001;21:947-53.

[5] Wada K, Nakamura K, Tamai Y, Tsuji M, Kawachi T, Hori A, et al. Soy isoflavone intake and breast cancer risk in Japan: from the Takayama study. Int J Cancer. 2013;133:952-60.

[6] Zamora-Ros R, Knaze V, Lujan-Barroso L, Kuhnle GG, Mulligan AA, Touillaud M, et al. Dietary intakes and food sources of phytoestrogens in the European Prospective Investigation into Cancer and Nutrition (EPIC) 24-hour dietary recall cohort. Eur J Clin Nutr. 2012;66:932-41.

[7] Setchell KD. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr. 1998;68:1333S-46S.

[8] Nagata C. Factors to consider in the association between soy isoflavone intake and breast cancer risk. J Epidemiol. 2010;20:83-9.

[9] Rafii F. The role of colonic bacteria in the metabolism of the natural isoflavone daidzin to equol. Metabolites. 2015;5:56-73.

[10] Atkinson C, Frankenfeld CL, Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. Exp Biol Med. 2005;230:155-70.

[11] Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr. 2002;132:3577-84.

[12] Zheng W, Dai Q, Custer LJ, Shu XO, Wen WQ, Jin F, et al. Urinary excretion of isoflavonoids and the risk of breast cancer. Cancer Epidemiol Biomarkers Prev. 1999;8:35-40.

[13] Wu AH, Yu MC, Tseng CC, Twaddle NC, Doerge DR. Plasma isoflavone levels versus self-reported soy isoflavone levels in Asian-American women in Los Angeles County. Carcinogenesis. 2004;25:77-81.

[14] Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-oestrogens and breast cancer. Lancet. 1997;350:990-4.

[15] Verheus M, van Gils CH, Keinan-Boker L, Grace PB, Bingham SA, Peeters PH. Plasma phytoestrogens and subsequent breast cancer risk. J Clin Oncol. 2007;25:648-55.

[16] Goodman MT, Shvetsov YB, Wilkens LR, Franke AA, Le Marchand L, Kakazu KK, et al. Urinary phytoestrogen excretion and postmenopausal breast cancer risk: the multiethnic cohort study. Cancer Prev Res. 2009;2:887-94.

[17] Grace PB, Taylor JI, Low YL, Luben RN, Mulligan AA, Botting NP, et al. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. Cancer Epidemiol Biomarkers Prev. 2004;13:698-708.

[18] Ward H, Chapelais G, Kuhnle GG, Luben R, Khaw KT, Bingham S, et al. Breast cancer risk in relation to urinary and serum biomarkers of phytoestrogen exposure in the European Prospective into Cancer-Norfolk cohort study. Breast Cancer Res. 2008;10:R32.

[19] Tseng M, Byrne C, Kurzer MS, Fang CY. Equol-producing status, isoflavone intake, and breast density in a sample of U.S. Chinese women. Cancer Epidemiol Biomarkers Prev. 2013;22:1975-83.

[20] Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, et al. Equol status modifies the association of soy intake and mammographic density in a sample of postmenopausal women. Cancer Epidemiol Biomarkers Prev. 2008;17:33-42.

[21] Schnitt SJ, Connolly JL. Pathology of benign breast disorders. In: Harris JR, Lippman ME, Morrow M, Osborne CK, editors. Diseases of the breast, Philadelphia: Lippincott, Williams & Wilkins; 2000, p. 75-93.

[22] Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, et al. Benign breast disease and the risk of breast cancer. N Engl J Med. 2005;353:229-37.

[23] Lampe JW, Nishino Y, Ray RM, Wu C, Li W, Lin MG, et al. Plasma isoflavones and fibrocystic breast conditions and breast cancer among women in Shanghai, China. Cancer Epidemiol Biomarkers Prev. 2007;16:2579-86.

[24] Thomas DB, Gao DL, Ray RM, Wang WW, Allison CJ, Chen FL, et al. Randomized trial of breast self-examination in Shanghai: final results. J Natl Cancer Inst. 2002;94:1445-57.

[25] Shannon J, King IB, Lampe JW, Gao DL, Ray RM, Lin MG, et al. Erythrocyte fatty acids and risk of proliferative and nonproliferative fibrocystic disease in women in Shanghai, China. Am J Clin Nutr. 2009;89:265-76.

[26] Aaman TB, Stalsberg H, Thomas DB. Extratumoral breast tissue in breast cancer patients: a multinational study of variations with age and country of residence in low- and high-risk countries. WHO Collaborative Study of Neoplasia and Steroid Contraceptives. Int J Cancer. 1997;71:333-9.

448 [27] Li W, Ray RM, Lampe JW, Lin MG, Gao DL, Wu C, et al. Dietary and other risk factors in
449 women having fibrocystic breast conditions with and without concurrent breast cancer: a nested
450 case-control study in Shanghai, China. Int J Cancer. 2005;115:981-93.
451 [28] Frankenfeld CL, Lampe JW, Shannon J, Gao DL, Ray RM, Prunty J, et al. Frequency of soy
452 food consumption and serum isoflavone concentrations among Chinese women in Shanghai.
453 Public Health Nutr. 2004;7:765-72.
454 [29] Setchell KD, Cole SJ. Method of defining equol-producer status and its frequency among
455 vegetarians. J Nutr. 2006;136:2188-93.
456 [30] Wu C, Ray RM, Lin MG, Gao DL, Horner NK, Nelson ZC, et al. A case-control study of
457 risk factors for fibrocystic breast conditions: Shanghai Nutrition and Breast Disease Study, China,
458 1995-2000. Am J Epidemiol. 2004;160:945-60.
459 [31] Shannon J, Ray R, Wu C, Nelson Z, Gao DL, Li W, et al. Food and botanical groupings and
460 risk of breast cancer: a case-control study in Shanghai, China. Cancer Epidemiol Biomarkers
461 Prev. 2005;14:81-90.
462 [32] Virk-Baker MK, Barnes S, Krontiras H, Nagy TR. S-(-)equol producing status not
463 associated with breast cancer risk among low isoflavone-consuming US postmenopausal women
464 undergoing a physician-recommended breast biopsy. Nutr Res. 2014;34:116-25.
465 [33] Niculescu MD, Pop EA, Fischer LM, Zeisel SH. Dietary isoflavones differentially induce
466 gene expression changes in lymphocytes from postmenopausal women who form equol as
467 compared with those who do not. J Nutr Biochem. 2007;18:380-90.
468 [34] Verheus M, van Gils CH, Kreijkamp-Kaspers S, Kok L, Peeters PH, Grobbee DE, et al. Soy
469 protein containing isoflavones and mammographic density in a randomized controlled trial in
470 postmenopausal women. Cancer Epidemiol Biomarkers Prev. 2008;17:2632-8.

[35] Maskarinec G, Morimoto Y, Heak S, Isaki M, Steinbrecher A, Custer L, et al. Urinary estrogen metabolites in two soy trials with premenopausal women. Eur J Clin Nutr. 2012;66:1044-9.

[36] Travis RC, Spencer EA, Allen NE, Appleby PN, Roddam AW, Overvad K, et al. Plasma phyto-oestrogens and prostate cancer in the European Prospective Investigation into Cancer and Nutrition. Br J Cancer. 2009;100:1817-23.

[37] Hernandez BY, McDuffie K, Franke AA, Killeen J, Goodman MT. Reports: plasma and dietary phytoestrogens and risk of premalignant lesions of the cervix. Nutr Cancer. 2004;49:109-24.

[38] Kurahashi N, Iwasaki M, Inoue M, Sasazuki S, Tsugane S. Plasma isoflavones and subsequent risk of prostate cancer in a nested case-control study: the Japan Public Health Center. J Clin Oncol. 2008;26:5923-9.

[39] Shimazu T, Inoue M, Sasazuki S, Iwasaki M, Sawada N, Yamaji T, et al. Plasma isoflavones and the risk of lung cancer in women: a nested case-control study in Japan. Cancer Epidemiol Biomarkers Prev. 2011;20:419-27.

[40] Ko KP, Park SK, Park B, Yang JJ, Cho LY, Kang C, et al. Isoflavones from phytoestrogens and gastric cancer risk: a nested case-control study within the Korean Multicenter Cancer Cohort. Cancer Epidemiol Biomarkers Prev. 2010;19:1292-300.

[41] Liu B, Qin L, Liu A, Uchiyama S, Ueno T, Li X, et al. Prevalence of the equol-producer phenotype and its relationship with dietary isoflavone and serum lipids in healthy Chinese adults. J Epidemiol. 2010;20:377-84.

[42] Frankenfeld CL, Atkinson C, Thomas WK, Gonzalez A, Jokela T, Wahala K, et al. High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart. Br J Nutr. 2005;94:873-6.

[43] Setchell KD, Brown NM, Summer S, King EC, Heubi JE, Cole S, et al. Dietary factors influence production of the soy isoflavone metabolite s-(-)equol in healthy adults. J Nutr. 2013;143:1950-8.

[44] Franke AA, Lai JF, Halm BM, Pagano I, Kono N, Mack WJ, et al. Equol production changes over time in postmenopausal women. J Nutr Biochem. 2012;23:573-9.

[45] Franke AA, Lai JF, Pagano I, Morimoto Y, Maskarinec G. Equol production changes over time in pre-menopausal women. Br J Nutr. 2012;107:1201-6.

Table 1. Selected characteristics of controls, women with fibrocystic breast conditions and breast cancer cases [a]

	Controls	Fibrocystic Breast Conditions			Breast Cancer Cases		
		Non-proliferative	Proliferative	All	Non-proliferative	Proliferative	All
	n=1027	n=130[b]	n=172[b]	n=443[b]	NCT[c] n=99[b]	NCT n=92[b]	n=269[b]
Age (years)							
≤ 39	13 (1.3)	17 (13.1)	16 (9.3)	57 (12.9)	3 (3.0)	4 (4.3)	9 (3.3)
40-44	457 (44.5)	54 (41.5)	81 (47.1)	198 (44.7)	27 (27.3)	24 (26.1)	75 (27.9)
45-49	215 (20.9)	33 (25.4)	46 (26.7)	117 (26.4)	19 (19.2)	25 (27.2)	56 (20.8)
50-59	121 (11.8)	14 (10.8)	9 (5.2)	28 (6.3)	21 (21.2)	11 (12.0)	40 (14.9)
≥ 60	221 (21.5)	12 (9.2)	20 (11.6)	43 (9.7)	29 (29.3)	28 (30.4)	89 (33.1)
Number of live births							
None	37 (3.6)	7 (4.9)	8 (3.8)	20 (3.7)	6 (5.9)	6 (6.2)	16 (5.2)
1	694 (67.8)	95 (62.5)	137 (68.5)	350 (66.4)	49 (63.4)	57 (70.8)	145 (66.8)
2	119 (11.6)	12 (9.4)	12 (12.0)	30 (10.5)	23 (15.5)	9 (7.4)	44 (12.3)
≥ 3	173 (16.9)	14 (23.2)	15 (15.8)	40 (19.3)	21 (15.2)	20 (15.7)	64 (15.7)

Age at first live birth (years)														
≤ 24	258	(26.3)	23	(26.3)	29	(25.8)	68	(25.9)	30	(24.5)	26	(24.0)	84	(25.0)
25-29	582	(58.9)	79	(60.2)	106	(59.9)	283	(60.5)	43	(52.9)	40	(51.3)	120	(54.2)
≥ 30	146	(14.8)	19	(13.5)	29	(14.3)	68	(13.6)	18	(22.6)	20	(24.7)	47	(20.8)
Months of breast feeding														
Never	174	(17.7)	20	(17.1)	33	(16.8)	83	(18.1)	14	(16.8)	16	(21.4)	38	(17.3)
≤ 6	203	(20.7)	30	(24.2)	50	(29.2)	118	(26.1)	20	(24.4)	16	(18.2)	50	(21.5)
7-12	352	(36.0)	44	(28.5)	50	(28.3)	146	(29.6)	26	(32.5)	30	(39.9)	83	(38.0)
13-24	110	(11.3)	13	(14.0)	12	(10.5)	31	(11.0)	18	(15.3)	6	(5.5)	33	(10.5)
≥ 25	139	(14.3)	10	(16.2)	15	(15.2)	139	(14.3)	14	(11.0)	18	(15.0)	48	(12.8)
Duration of oral contraceptive use														
Never used	939	(91.5)	110	(83.4)	155	(88.1)	395	(87.1)	87	(86.9)	80	(87.6)	240	(89.8)
≤ 1 year	34	(3.3)	10	(9.3)	8	(4.2)	26	(6.5)	8	(8.7)	7	(8.0)	16	(6.2)
> 1 year	53	(5.2)	10	(7.3)	9	(7.7)	21	(6.5)	4	(4.4)	5	(4.4)	13	(4.0)

Age at first menstrual period (years)														
≤ 13	163	(15.9)	28	(16.7)	25	(14.0)	84	(16.7)	24	(28.1)	15	(13.4)	53	(21.7)
14	200	(19.5)	29	(23.1)	44	(22.5)	101	(21.4)	16	(19.5)	17	(21.7)	53	(22.3)
15	204	(19.9)	26	(20.4)	35	(19.8)	96	(21.1)	24	(21.7)	20	(23.5)	62	(22.1)
16	213	(20.8)	22	(14.0)	32	(19.2)	73	(16.3)	17	(17.0)	15	(13.8)	44	(14.8)
≥ 17	246	(24.0)	25	(25.8)	36	(24.4)	88	(24.6)	18	(13.7)	25	(27.6)	57	(19.1)
Menopause														
Yes	357	(34.8)	25	(32.0)	37	(34.0)	80	(32.9)	50	(36.5)	39	(32.4)	127	(33.7)
Prior breast lumps														
Yes	31	(3.1)	10	(6.7)	24	(13.5)	48	(9.8)	5	(6.3)	8	(8.6)	16	(7.2)
Times breast self-examination per year														
Never	697	(68.1)	47	(41.1)	70	(44.3)	169	(41.6)	51	(51.0)	51	(51.0)	140	(49.6)
1-6	135	(13.2)	21	(11.4)	26	(12.6)	73	(14.0)	17	(19.9)	18	(19.4)	55	(21.5)
7-12	186	(18.2)	59	(45.2)	70	(40.0)	185	(41.2)	29	(27.7)	22	(27.8)	68	(26.9)
≥ 13	6	(0.6)	3	(2.3)	6	(3.1)	14	(3.2)	2	(1.5)	1	(1.9)	5	(2.0)

Education														
Elementary school or less	193	(18.9)	11	(17.6)	14	(14.2)	35	(16.3)	30	(21.7)	20	(15.7)	79	(19.8)
Middle school	803	(78.2)	110	(75.5)	149	(80.0)	384	(77.9)	63	(74.6)	64	(76.5)	172	(74.7)
College	30	(2.9)	9	(6.9)	9	(5.8)	23	(5.7)	6	(3.7)	8	(7.8)	18	(5.5)
Body mass index (kg/m2)														
≤ 20	192	(18.7)	39	(26.0)	36	(16.0)	116	(21.2)	16	(18.0)	17	(18.4)	43	(16.3)
21-25	603	(58.7)	72	(56.5)	101	(58.8)	253	(57.1)	61	(62.0)	50	(56.9)	158	(61.2)
> 25	232	(22.6)	19	(17.5)	35	(25.2)	73	(21.6)	22	(20.0)	25	(24.7)	68	(22.5)

[a] Data are shown as n (%); total numbers per variable may not add up to the total number of women per column due to some missing data

[b] Indirect age-adjusted percentages based on age distribution of the controls

[c] NCT = non-cancerous tissue

Table 2. Plasma equol concentration among controls, women with fibrocystic breast conditions (FBC) and breast cancer (BC) cases by age group

	Controls		FBC cases		BC cases	
	n	Geometric mean (95% CI)[a]	n	Geometric mean (95% CI)[a]	n	Geometric mean (95% CI)[a]
All ages combined	1027	6.87 (6.25, 7.55)	443	5.45 (4.76, 6.24)	269	5.59 (4.73, 6.59)
Age ≤39	13	7.83 (3.16, 19.40)	57	5.02 (3.27, 7.71)	9	2.88 (0.88, 9.44)
Age 40-44	457	6.81 (5.90, 7.85)	198	5.31 (4.33, 6.50)	75	6.41 (4.67, 8.79)
Age 45-49	215	5.79 (4.75, 7.06)	117	5.71 (4.42, 7.38)	56	4.68 (3.28, 6.67)
Age 50-59	121	6.39 (5.02, 8.14)	28	4.02 (2.52, 6.41)	40	4.65 (3.08, 7.02)
Age ≥60	221	8.53 (6.89, 10.6)	43	7.41 (4.92, 11.2)	89	6.46 (4.85, 8.59)

[a] data presented as geometric mean and 95% confidence interval; plasma equol concentration in nmol/L

Table 3. Fibrocystic breast conditions (FBC) and breast cancer in relation to plasma equol concentrations

	Number of women (%)			FBCs vs. controls		Breast cancer vs. controls		Breast cancer vs. FBCs	
	Control	FBC	Cancer	OR[a]	95% CI	OR[a]	95% CI	OR[a]	95% CI
Equol (nmol/L)									
Non-proliferative NCT[b]									
< 20	796 (77.5)	107 (82.3)	86 (86.9)	1.00	Ref.	1.00	Ref.	1.00	Ref.
20 to <45	114 (11.1)	13 (10.0)	6 (6.1)	1.27	0.59-2.74	0.67	0.26-1.74	0.69	0.24-2.03
≥45	117 (11.4)	10 (7.7)	7 (7.1)	1.25	0.54-2.90	0.93	0.36-2.40	0.80	0.26-2.49
p trend					0.49		0.64		0.53
Proliferative NCT (including atypia)									
< 20	796 (77.5)	143 (83.1)	74 (80.4)	1.00	Ref.	1.00	Ref.	1.00	Ref.
20 to <45	114 (11.1)	18 (10.5)	9 (9.8)	1.09	0.51-2.32	0.94	0.38-2.32	1.28	0.51-3.20
≥45	117 (11.4)	11 (6.4)	9 (9.8)	0.70	0.28-1.74	1.88	0.75-4.70	1.73	0.59-5.06
p trend					0.57		0.26		0.28

Total									
< 20	796 (77.5)	362 (81.7)	223 (82.9)	1.00	Ref.	1.00	Ref.	1.00	Ref.
20 to <45	114 (11.1)	48 (10.8)	27 (10.0)	1.05	0.61-1.80	1.06	0.59-1.83	0.97	0.56-1.67
≥45	117 (11.4)	33 (7.4)	19 (7.1)	0.84	0.46-1.53	1.18	0.61-2.29	0.91	0.47-1.74
p trend					0.64		0.61		0.76

[a] Data are presented as odds ratios (ORs) and 95% confidence intervals (CIs); ORs were adjusted for age, plasma genistein (quartiles), and lab method for genistein analysis, and included a strata variable for blood draw year

[b] NCT = non-cancerous tissue

Table 4. Fibrocystic breast conditions (FBC) and breast cancer in relation to log_{10} plasma equol:daidzein ratio among women with plasma daidzein concentration ≥20 nmol/L

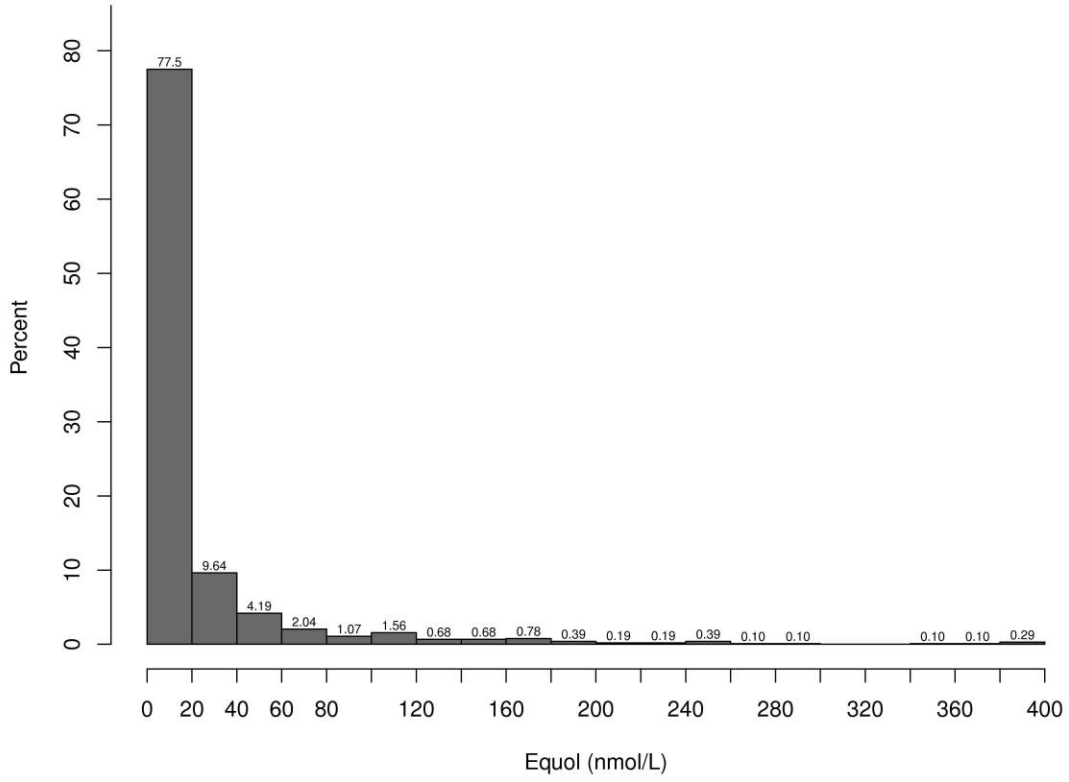
	Number of women (%)			FBCs vs. controls		Breast cancer vs. controls		Breast cancer vs. FBCs	
	Control	FBC	Cancer	OR[a]	95% CI	OR[a]	95% CI	OR[a]	95% CI
Log_{10} plasma equol:daidzein ratio									
Non-proliferative NCT[b]									
-3.35 to -1.43	258 (33.3)	20 (29.0)	19 (30.6)	1.00	Ref.	1.00	Ref.	1.00	Ref.
-1.42 to -0.77	258 (33.3)	24 (34.8)	28 (45.2)	1.02	0.48-2.13	1.76	0.89-3.47	1.36	0.83-6.71
-0.76 to 1.96	258 (33.3)	25 (36.2)	15 (24.2)	1.14	0.54-2.39	0.87	0.40-1.88	1.09	0.36-3.28
p trend					0.37		0.48		0.40
Continuous log_{10} plasma equol:daidzein ratio				1.21	0.79-1.84	0.91	0.60-1.37	0.78	0.42-1.46
p-value					0.38		0.66		0.44

Proliferative NCT (including atypia)									
-3.35 to -1.43	258 (33.3)	27 (26.5)	13 (20.3)	1.00	Ref.	1.00	Ref.	1.00	Ref.
-1.42 to -0.77	258 (33.3)	48 (47.1)	35 (54.7)	2.06	1.08-3.93	2.95	1.37-6.35	1.37	0.59-3.22
-0.76 to 1.96	258 (33.3)	27 (26.5)	16 (25.0)	1.05	0.51-2.16	1.36	0.58-3.23	1.36	0.51-3.67
p trend					0.81		0.31		0.31
Continuous log_{10} plasma equol:daidzein ratio				1.13	0.76-1.68	1.38	0.88-2.15	1.37	0.77-2.42
p-value					0.56		0.16		0.28
Total									
-3.35 to -1.43	258 (33.3)	78 (27.8)	45 (25.6)	1.00	Ref.	1.00	Ref.	1.00	Ref.
-1.42 to -0.77	258 (33.3)	115 (40.9)	82 (46.6)	1.57	0.99-2.51	2.37	1.43-3.95	1.44	0.85-2.43
-0.76 to 1.96	258 (33.3)	88 (31.3)	49 (27.8)	1.17	0.71-1.93	1.26	0.72-2.18	1.10	0.62-1.94
p trend					0.42		0.38		0.92
Continuous log_{10} plasma equol:daidzein ratio				1.19	0.90-1.58	1.20	0.89-1.62	0.95	0.69-1.33
p-value					0.23		0.24		0.78

[a] Data are presented as odds ratios (ORs) and 95% confidence intervals (CIs); ORs were adjusted for age and lab method for daidzein analysis, and included a strata variable for blood draw year

[b] NCT = non-cancerous tissue

Figure 1. Frequency distribution of plasma equol concentration among control women [a]



[a] n=1027